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MAGNETOSOMES, METHOD FOR MAKING AND USING

Field of invention

The invention relates to specific magnetosomes with magnetic particles of 5 maximally 43 – 45 nm, and method for making and using them. The invention also relates to magnetoliposomes which can be obtained from these magnetosomes by liposomal encapsulation. The magnetoliposomes of the present invention are useful for medicinal applications.

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Background

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Superparamagnetic iron particles are known to be applied in medical diagnostics as NMR contrast agents or in the form of immunoconjugates or as synthetic drug carriers. Matsunaga et al. described in 1989 magnetosomes obtained from the magnetic bacterium *Magnetospirillum* spec. ABMI (JP7-241192-A) and their use. However, these magnetosomes have the disadvantage that they are comparatively large, thus bringing about the danger of embolisms.

Description of the invention

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The object of the present invention is to provide specific magnetosomes which are smaller than those known, thus improving their medical use for reaching the envisaged targets in the body of the patient and also with reduced danger of embolisms.

It was detected that magnetosomes with magnetic particles < 50 nm are contained in the bacterium *Magnetospirillum gryphiswaldense*. To our surprise, it was possible to produce these specific magnetosomes of the magnetic bacterium 5 *Magnetospirillum gryphiswaldense* on a semi-industrial scale.

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Accordingly, the object of the invention are the magnetosomes themselves, their method of preparation and their use in medicine and pharmacy.

10 The magnetosomes of the present invention contain a magnetic oxide magnetite Fe_3O_4 monocrystal with a maximum diameter of 43 – 45 nm surrounded by a phospholipid membrane. As a rule, they have a cubooctahedral shape.

15 The membrane is suitably phosphatidyl ethanolamine, phosphatidyl glycerol and phosphatidyl choline containing mainly the fatty acids palmitic acid, palmitoleic acid and oleic acid. The membrane suitably contains 53 ± 6 % phosphatidyl ethanolamine, 38 ± 6 % phosphatidyl glycerol and 8.9 ± 5 % phosphatidyl choline where mainly the fatty acids palmitic acid (approx. 18.4 %), palmitoleic acid (approx. 25.6 %) and oleic acid (approx. 45.9 %) can be found.

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A suitable embodiment of the present invention contains the magnetosomes as chains up to 100, most suitably 10-60 magnetosomes and with a cationic surface charge.

This chain form of magnetosomes increases the probability that antibodies and therapeutic agents will be correctly bound to them and become effective.

In addition, these are also magnetosomes with additionally covalently bound
5 antibodies or therapeutic agents bound to the magnetosome membrane through
respective reactive groups.

The invention also comprises a method for preparing these new magnetosomes. They are isolated from the magnetic bacterium *Magnetospirillum gryphiswaldense*
10 according to a new fermentation method. For this purpose a new simple culture medium of 0.3 g of KH_2PO_4 , 1 g of Na acetate, 1 g of a soybean peptone (sold by Merck), 0.1 g of NH_4Cl , 0.1 g of yeast extract, at pH 6.9, which does not contain a
complexing agent for iron is suitably used. The concentration of oxygen in the medium
is maintained below 2 %, later Na acetate and FeSO_4 are added. After approx. 30
15 hours the magnetic cells can be gathered. After subjecting the cells to a lysis the magnetosomes are obtained in a high output according to a new method by separating them from cell fragments and cell sap in a magnetic separation column by a strong,
powerful permanent magnet (Sm-Neodyn) and purifying them by washing.

20 Furthermore, magnetosomes according to the present invention are packed in liposomes, forming themselves liposomes with other lipids or bound to the surface of liposomes. Such liposomes are

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- (i) classical liposomes (MLV, SUV, LUV);
- (ii) stealth liposomes (PEG);
- (iii) micellar systems (e.g. SDS, triton, sodium cholate);
- (iv) immunoliposomes containing e.g. antibodies or fab fragments
against antigens associated with diseases or adhesion
molecules bound to the surface of the liposomes;
- (v) cationic liposomes (DAG-Chol, DOCSPER); and
- (vi) fusogenic liposomes (reconstituted fusion proteins in liposomes).

10 Magnetoliposomes are prepared according to methods known *per se*, as e.g.
described in German patents Nos. 41 34 158; 44 30 593; 44 46 937; and 196 31 189
with the magnetosomes being suitably added to the initial lipids.

15 The suitable modifications of magnetoliposomes and magnetosomes according to
the present invention are represented in Table 1 below.

Table 1

Magnetoliposomes					
20	Classical	Stealth	Immuno	Cationic	Fusogenic
				e.g.	e.g.
	MLV	PEG	anti-CEA	DAC Chol/DOPE	HN, F protein
	SUV		anti Thy1.1	SP Chol/DOPE	(Sendai virus)

5	LUV (REV)	anti CD44 anti CD54 anti CD56 anti CD30 anti CD31	DAC-Quat. Chol/DOPE DOCSPER	→ [pH 7] synthetic fusion proteins HA influenza virus [pH 5,2] Cochleates
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Magnetosomes

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Immuno	Gene or antisense oligonucleotide
anti CEA	or ribozyme modified
anti CD44	
anti CD 54, CD56	
anti CD30	

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The magnetosomes and magnetoliposomes according to the invention can contain specific antibodies and one or a few therapeutic agents chemically coupled to their surfaces and enclosed, i.e. encapsulated radionuclides.

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In addition, they, together with genetic material such e.g. plasmids, therapy genes, antisense oligonucleotides, ribozymes or gene diagnostic agents, can form cationic complexes suited for the transfer of genes.

These magnetosomes and magnetoliposomes (these terms being used

interchangeably herein) according to the invention have a comprehensive spectrum of application. Owing to their magnetic properties they are used *per se* (also unmodified) as contrast agents for NMR examinations and as markers for mapping magnetic susceptibilities such as by a SQUID biomagnet meter, and also as diagnostic agents for 5 the detection of various diseases, and foci of inflammatory or therapeutic agents as e.g. for purging (taking out diseased cells), as diagnostic agents for tumoral diseases or in lymphography, for inflammatory processes, for multiple sclerosis, Alzheimer disease and for Parkinson's disease, or as a therapeutic agent against tumoral diseases, inflammatory processes, and metabolic diseases.

10 Diagnostic agents are suitably used in the form of immunomagnetosomes or immunomagnetoliposomes. For this, antibodies or fab fragments against antigens associated with diseases or adhesion molecules or ligands are covalently coupled to the magnetosome and magnetoliposome membrane through respective groups, suitably to 15 phosphatidyl ethanolamine contained in the membrane through spacers of differing lengths.

20 In particular, they are used as diagnostic agents for the detection of tumoral diseases or in lymphography, with among others anti CEA, anti CD44 being coupled to the magnetosome membrane or magnetoliposome membrane as a reagent.

These antibody coupling products are also suited for detecting inflammatory

processes such as arthroses (suitably with anti CD54, anti CD56) or for detecting multiple sclerosis or Alzheimer's disease (suitably anti- β -amyloid, anti APOE4), Hogkin lymphoma cells (suitably with anti CD30) and Parkinson's disease.

5 The magnetosomes according to the invention are particularly well suited for diagnostic applications.

10 It is necessary to use magnetoliposomes simultaneously to bring a therapeutic substance in relevant quantities to the target location. They are not only suited for coupling but also for enclosing therapeutic agents. In the case of magnetosomes therapeutic agents can be coupled only with a spacer being interconnected.

15 According to the present invention an essential possibility of use is that therapeutic agents are coupled (magnetosomes) or coupled or enclosed (magnetoliposomes). These therapeutic agents can be enclosed in the membrane or in the aqueous interior of the liposomes depending on lipophilicity or hydrophilicity.

Thus, the following suitable coupling variants are obtained according to the invention:

20 the therapeutic agent(s) is (are) coupled to the magnetosome or enclosed in the membrane;

 the therapeutic agent(s) is (are) coupled to the magnetosome or enclosed in the

membrane and packed in liposomes;

the magnetosome is packed as liposome and this or the therapeutic agents are enclosed in the aqueous interior of the liposomes; and

therapeutic agents are coupled to the magnetosome or enclosed in the
5 membrane, the magnetosome is packed in liposomes and at least one further
therapeutic agent is enclosed in the aqueous or lipophilic interior of the liposomes.

Important therapeutic agents that can be considered for this purpose include
chemotherapeutic agents such as carboplatin or taxol, and radiotherapeutic agents such
10 as yttrium, iodine, technetium or boron, and also therapy genes such as suicide genes,
antisense oligonucleotides, ribozymes or cytokine genes can be coupled in this manner.

The invention enables a broad scope of medical application. The essential
advantage of the magnetosomes and magnetoliposomes according to the invention
15 enables that metastases can be better reached in the body and detected early, their
enrichment in the lymphatic vessels is improved, and blood-brain barriers are better
overcome by the new particles which is of particular importance to the detection of
Alzheimer's plaques and the diagnosis of brain tumors.

20 The invention is explained in greater detail by the following examples of thereof.

Example 1

Obtaining magnetosomes

To obtain magnetosomes in masses the cells of the magnetic bacterium

5 *Magnetospirillum gryphiswaldense* were bred in a 100 ℓ fermenter (LP 352, Bioeng. AG) at 30°C in a culture medium of the following composition (per 1000 m ℓ): 0.3 g of KH₂PO₄, 1 g of Na acetate, 1 g of soybean peptone (Merck), 0.1 g of NH₄Cl, 0.1 g of yeast extract, pH 6.9. Inoculation was effected by adding 5 ℓ of pre-culture to 70 ℓ of the medium. Aeration was regulated by stirring and input of compressed air so that 10 the concentration of oxygen in the medium did not exceed 2 % of saturation. 70 g of Na acetate and iron sulfate were added to a concentration of 100 μ M with the OD₄₀₀ = 0.55. After approx. 30 hours it was possible to gather magnetic cells.

15 The cells were centrifuged and washed. After the cell extract passed the French press three times and was subsequently subjected to a low-run centrifuging it was put into 20 mM HEPES/4 mM EDTA through a magnetic separation column (Miltenyi Biotec). The column was exposed to the magnetic field of a strong permanent magnet (Sm-Neodyn) to separate the magnetic particles. This produced a strong 20 inhomogeneous magnetic field in a magnetizable column material for a specific binding of the magnetic particles. The magnetosomes were washed in the column with 20 mM HEPES/200 mM NaCl to remove specifically associated pollution. After having been washed with 20 mM HEPES the magnetosomes were flushed from the column after

removing the magnetic field. To separate potentially available membrane contaminations, the magnetosome suspension was applied to a two-layer (50/55 % saccharose) sugar gradient and centrifuged in an ultracentrifuge with 25,000 rpm for 25 hours. Potentially contained membrane components accumulated at the buffer-
5 saccharose solution interphase whereas the magnetosome particles appeared as pellets on the bottom of the tube. The magnetosomes thus obtained appeared to be electronmicroscopically pure and showed a distinct lipid and protein pattern.

Example 2

Use of magnetosomes

There were used magnetosomes from *M. gryphiswaldense* with an iron content of 1.35 g Fe/ℓ (determined by atomic absorption spectroscopy (AAS)). The relaxivities were determined by means of a Bruker Minispec pc 120 at 37°C and 0.47 T as:

$$R_1 = 25.503 \text{ mM}^{-1} \cdot \text{s}^{-1}$$

$$R_2 = 226.179 \text{ mM}^{-1} \cdot \text{s}^{-1}.$$

The relaxivities, in particular R_2 , are high as compared with various SPIOs (superparamagnetic iron oxide formulations). Comparable values were obtained only for SPIO-SUVs (small unilamellar vesicles).

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The following *in vivo* experiment was carried out: The remaining substance quantity (0.4 mℓ) was injected *in vivo* into the tail vein of a male WAG/RIJ (270 g rat)

with a CC531 adenocarcinoma implanted into the liver. Thus, the animal received magnetosomes in a dose of $35.81 \mu\text{mol Fe/kg}$ of rat weight. The NMR examination was carried out with a Bruker Biospec BMT 24/40 instrument. Thereby, before, immediately after the infection and then at the time indicated in Table 2 nine 3 mm layers and an enclosed external standard tube with the RARE sequence (TR = 2500 ms, TE = 20 ms, RF = 8; NE = 8) were taken up through the abdomen of the rat. The signal intensities in the liver and the tumour were measured in four different layers and were evaluated. The indicated weakening of the relative signal intensity SI_{rel} is calculated as follows:

$$\begin{aligned} \text{SI}_{\text{rel}} &= (\text{SI}_{\text{post lip}}/\text{SI}_{\text{standard}})/(\text{SI}_{\text{pre lip}}/\text{SI}_{\text{standard}}) \\ \text{SI}_{\text{pre lip}} &= \text{signal intensity before applying liposomes} \\ \text{SI}_{\text{post lip}} &= \text{signal intensity after applying liposomes} \\ \text{SI}_{\text{standard}} &= \text{signal intensity of the standard.} \end{aligned}$$

Given this comparatively low dose a signal reduction, up to already 90 % was reached in the liver, however in the tumor only weak SI reductions were observed (Table 2). This means that the tumor clearly stands out against the healthy liver tissue (Fig. 1).

Table 2

	Tumor mean from all > layers				Mean	
5	pre	1.00	1.00	1.00	1.00	0.00
	5 min.	0.93	0.89	0.91	0.98	0.04
	15 min.	1.00	0.96	0.98	1.02	0.03
	31 min.	1.04	0.99	0.99	1.06	0.04
	48 min.	1.01	0.98	0.98	1.06	0.04
	65 min.	1.00	0.98	0.98	1.13	0.07
	82 min.	0.95	0.94	0.93	1.05	0.06
	113 min.	0.93	0.86	0.91	1.10	0.10
	24 h	0.93	0.86	0.91	1.10	0.10
	15 48 h	1.05	1.00	1.02	1.14	0.10
Liver						
20					Mean	Standard deviation
	pre	1.00	1.00	1.00	1.00	0.00
	5 min.	0.18	0.29	0.13	0.12	0.08
	15 min.	0.19	0.35	0.10	0.13	0.11
	31 min.	0.13	0.19	0.09	0.15	0.04
	48 min.	0.18	0.14	0.14	0.23	0.04
	65 min.	0.24	0.18	0.14	0.13	0.05
	82 min.	0.23	0.13	0.11	0.17	0.05
	30 113 min.	0.13	0.13	0.11	0.17	0.03
	24 h	0.11	0.13	0.11	0.17	0.03
	48 h	0.11	0.12	0.12	0.11	0.01